



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>A01N 63/00, C12N 5/10, 15/09</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 00/18239</b> <b>(43) International Publication Date:</b> 6 April 2000 (06.04.00)
<b>(21) International Application Number:</b> PCT/US99/22207 <b>(22) International Filing Date:</b> 24 September 1999 (24.09.99) <b>(30) Priority Data:</b> 60/101,812 25 September 1998 (25.09.98) US <b>(71) Applicants (for all designated States except US):</b> UNIVERSITY OF NEBRASKA BOARD OF REGENTS [US/US]; Regents Hall, 3835 Holdrege Street, Lincoln, NE 68598 (US). HARVARD-MIT DIVISION OF HEALTH SCIENCE AND TECHNOLOGY [US/US]; Cambridge, MA (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> FOX, Ira, J. [US/US]; 600 S. 42nd Street, Omaha, NE 68198 (US). LABOULCH, Phillippe [US/US]; Cambridge, MA (US). <b>(74) Agents:</b> REED, Janet, E. et al.; Dann, Dorfman, Herrell and Skillman, Suite 720, 1601 Market Street, Philadelphia, PA 19103 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> REVERSIBLY IMMORTALIZED HEPATOCYTES AND METHODS OF USE  <b>(57) Abstract</b>  Hepatocyte cells that are reversibly immortalized and grown in culture, which are functional and safe for use in transplantation, are disclosed. Also disclosed are methods for immortalizing primary hepatocytes, expanding the population of immortalized hepatocytes in culture, then reversing the immortalization to produce hepatocytes that are functional and safe for use in transplantation.		

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REVERSIBLY IMMORTALIZED HEPATOCYTES  
AND METHODS OF USE

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10

FIELD OF THE INVENTION

This invention relates to the treatment of liver disease and hepatic failure. In particular, the invention provides hepatocyte cells reversibly immortalized and grown in culture, which are functional and safe for use in transplantation.

BACKGROUND OF THE INVENTION

Several scientific publications are referenced in this patent application to describe the state of the art to which the invention pertains. Each of these references is incorporated by reference herein, in its entirety.

Whole-organ liver transplantation is the current method of choice for treating patients with hepatic failure. Recent experimental evidence indicates that hepatocyte transplantation instead of whole organ transplantation can be used to treat hepatic failure and liver-based metabolic diseases, thereby obviating in some cases the need for surgery, with its associated risk.

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Hepatocyte transplantation also could be useful as a temporary treatment for patients with chronic liver failure, who are awaiting whole-organ transplantation. The first unequivocally successful heptaocyte  
5 transplantation into a human patient was recently reported (Fox et al., New Eng. J. Med. 338: 1422-1426, 1998), thereby demonstrating the clinical feasibility of this treatment method.

The shortage of human livers available as a  
10 source of hepatocytes for transplantation severely limits the use of this method for the treatment of liver failure. Animal hepatocytes could serve as an alternative, but significant concerns would exist with respect to transmission of infectious agents and  
15 immunological or physiological incompatibility with human hosts.

Another alternative to the transplantation of primary hepatocytes is the use of a clonal hepatocyte cell line that could be grown in culture and would  
20 exhibit the characteristics of differentiated, non-transformed hepatocytes following transplantation. Cloned hepatocyte cell lines have been developed by immortalization with a temperature-sensitive SV40 large T antigen (SV40Tag) (Fox et al., Hepatology 21: 837-845,  
25 1995). These cells proliferate at the permissive temperature of 33°C and lack characteristic features of differentiated hepatocytes. At the non-permissive temperature (37-39°C), cell proliferation cease and the cells regain morphological characteristics of  
30 differentiated hepatocytes.

The aforementioned conditionally immortalized hepatocytes were found to function as well as primary hepatocytes following transplantation in rodents to reverse hyperammonemia-induced hepatic encephalopathy  
35 (Schumacher et al., Hepatology 24: 337-343, 1996) and to

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improve survival in experimentally-induced acute liver failure (Nakamura et al., Transplantation 63: 1541-1547, 1997). However, the continued presence of the oncogene (encoding SV40Tag) in these cells is of concern, inasmuch  
5 as it may increase the risk of malignant transformation following transplantation. A means to minimize or eliminate this risk heretofore has been unavailable.

#### SUMMARY OF THE INVENTION

10 According to one aspect of the invention, a method of making a population of functional hepatocytes for transplantation into a patient is provided. The method comprises: (a) providing a sample of primary hepatocytes; (b) immortalizing the hepatocytes by  
15 transforming the hepatocytes with a vector comprising a removable DNA segment containing an oncogene, thereby producing immortalized hepatocytes; (c) growing the immortalized hepatocytes; and (d) removing the oncogene from the immortalized hepatocytes, the removal resulting  
20 in the production of the population of functional hepatocytes for transplantation into the patient. Preferably, the hepatocytes are obtained from a human donor and the oncogene is a gene encoding SV40 large T antigen.

25 The oncogene is made removable by flanking it with recombinase target sites, and the removing is accomplished by introducing into the immortalized cells a gene that is expressed to produce a recombinase that specifically recognizes the recombinase target sites.  
30 Preferably, the recombinase is Cre recombinase and the recombinase target sites are loxP sites.

In preferred embodiments, the removable DNA segment further contains a suicide gene, which encodes a gene product that enables destruction of the immortalized  
35 cells by an exogenous agent if the removable DNA segment

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is not removed from the cells. the suicide gene preferably is a gene encoding herpes simplex virus thymidine kinase, and the cells are destroyed by exposure to gancyclovir if the removable DNA segment is not removed from the cells.

Another aspect of the invention provides a method of making a population of functional hepatocytes for transplantation into a patient, which comprises: (a) providing a sample of primary hepatocytes; (b) immortalizing the hepatocytes by transforming the hepatocytes with a vector comprising a removable DNA construct containing an oncogene, a selectable marker gene, and a gene encoding herpes simplex virus thymidine kinase, the genes together being flanked on either side by loxP sites; (c) growing the immortalized hepatocytes; and (d) reversing the immortalization of the hepatocytes by removing the DNA construct from the immortalized hepatocytes, the removing being accomplished by introducing into the immortalized hepatocytes a gene encoding Cre recombinase to effect excision of the DNA construct at the loxP sites, the excision resulting in the production of the population of functional hepatocytes for transplantation into the patient.

Populations of functional hepatocytes produced by the aforementioned methods are also provided, along with a method of treating a patient for hepatic failure, comprising transplanting into the patient a sufficient quantity of those hepatocytes to provide hepatic function to the patient.

According to another aspect of the invention, an immortalized hepatocyte is provided, which comprises a primary hepatocyte transformed with a DNA construct comprising two recombinase target sites that flank an oncogene which confers immortalization to the hepatocyte, wherein the immortalization is reversible by excision of

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the DNA construct by cleavage at the recombinase target sites when the target sites are exposed to a recombinase that specifically recognizes the target sites.

Preferably, the recombinase target sites are loxP sites and the immortalization is reversible by Cre recombinase cleavage at the loxP sites. The DNA construct further includes a selectable marker gene, and may further comprise a suicide gene, which encodes a gene product that enables destruction of the immortalized hepatocyte by an exogenous agent if the DNA construct is not removed from the cells. Preferably, the suicide gene is a gene encoding herpes simplex virus thymidine kinase, and the exogenous agent is gancyclovir.

In one embodiment, the primary hepatocyte is obtained from a human donor. In another embodiment the primary hepatocyte is obtained from a rat donor, and the immortalized cell line, C8-B, is provided.

According to another aspect of the invention, a reverse-immortalized hepatocyte that is functional upon transplantation into a patient is provided, which is produced by exposing the DNA construct within the above-described immortalized hepatocyte to a recombinase that excises the DNA construct by cleavage at the recombinase target sites. A method of treating a patient for hepatic failure is also provided, comprising transplanting into the patient a sufficient quantity of the reverse-immortalized hepatocytes of claim 24 to provide hepatic function to the patient.

Other features and advantages of the present invention will be understood by reference to the drawings, detailed description and examples that follow.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Schematic drawings of the integrating component of retroviral vector SSR69 before (upper) and

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after (lower) site-specific recombination. SSR69 contains the hygromycin B resistance gene (Hyg R) as a positive selectable marker and the herpes simplex virus thymidine kinase gene (HSV-TK) as a negative selectable marker. The Hyg R, HSV-TK and SV40 large T (SV40T) genes are flanked by loxP sites. The approximate locations of primers specific for SSR69 viral DNA (SSR-5' and SSR-3') and the neomycin resistance gene (NeoR, Neo-5' and Neo-3') are indicated. MoMLV, Moloney murine leukemia virus; LTR, long terminal repeat; IRES, internal ribosome entry.

Fig. 2. PCR and SQ-RT-PCR analysis of SSR69-immortalized hepatocytes (C8-B cells) before and after Ad-Cre infection. The PCR reaction used 4-fold serial dilutions of genomic DNA, isolated before (day 0) and 2 days after Ad-Cre infection (day 2). The SSR69 (SSR-5' and SSR-3') and NeoR-specific primers (which were used as a DNA loading control) are indicated in Fig. 1. SQ-RT-PCR reactions were performed using 4-fold serial dilutions of cDNA prepared with total RNA isolated before and 2, 5, and 7 days after Ad-Cre infection. Actin was used as a cDNA loading control and H<sub>2</sub>O was used as a PCR control. Triangles represent the degree of serial dilution of DNA or cDNA used in each PCR or SQ-RT-PCR reaction.

Fig. 3. Growth kinetics and <sup>3</sup>H-thymidine incorporation by C8-B cells. (A) The growth kinetics of C8-B cells were determined by plating cells at a density of 3X10<sup>4</sup> cells per well in 6-well plates. Cells were then infected (solid square) with Ad-Cre or mock infected (day 0). Each time point represents an average from triplicate samples. (B) <sup>3</sup>H-Thymidine incorporation was measured by incubating C8-B cells with 5 μCi/ml of <sup>3</sup>H-thymidine at 37°C for 2 hours before (day 0) and 1, 2, or 3 days after Ad-Cre infection. Results are shown as mean



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± SD from three independent experiments, each performed in triplicate.

**Fig. 4.** Expression of liver-specific genes by C8-B cells. Expression of the liver-specific genes, albumin (ALB), hepatocyte nuclear factor 4 (HNF4), UDP-glucuronosyltransferase-1 (UGT1), UDP-glucuronosyltransferase-2 (UGT2) and asialoglycoprotein receptor (ASGR), was analyzed by SQ-RT-PCR using total RNA isolated from C8-B cells before (day 0) and 2, 5, and 7 days after Ad-Cre infection. Four-fold serial cDNA dilutions were used in the PCR reactions. H<sub>2</sub>O was used as a negative PCR control, RNA isolated from primary rat hepatocytes was used as a positive control, and actin was used as cDNA loading control.

**Fig. 5.** Soft agar assay of C8-B/Ras cells. Anchorage independent growth of C8-B/Ras (SV40Tag<sup>+</sup>/ras<sup>+</sup>) cells was evaluated by soft agar assay. C8-B/Ras cells, mock infected or infected with Ad-Cre (indicated on the left) were incubated in the presence or absence of 5 μM gancyclovir (GCV, indicated on the top). Inserts are phase (40X) contrast micrographs of representative colonies.

## DETAILED DESCRIPTION OF THE INVENTION

### I. Definitions

Certain aspects of the present invention employ conventional molecular biology, microbiology, and recombinant DNA techniques that are well known in the art. See, e.g., Sambrook et al., "Molecular Cloning: A Laboratory Manual (1989); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription and Translation" [B.D. Hames & S.J. Higgins eds. (1984)]; "Animal Cell Culture" [R.I.

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Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984); or "Current Protocols in Molecular Biology", eds. Frederick M. Ausubel et al.,  
5 John Wiley & Sons, 1999.

Therefore, if appearing herein, the following terms have the definitions set out below.

A "coding sequence" or "coding region" refers to a nucleic acid molecule having sequence information  
10 necessary to produce a gene product, when the sequence is expressed.

The term "operably linked" or "operably inserted" means that the regulatory sequences necessary for expression of the coding sequence are placed in a  
15 nucleic acid molecule in the appropriate positions relative to the coding sequence so as to enable expression of the coding sequence. This same definition is sometimes applied to the arrangement other transcription control elements (e.g. enhancers) in an  
20 expression vector.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the  
25 expression of a coding sequence in a host cell.

The terms "promoter", "promoter region" or "promoter sequence" refer generally to transcriptional regulatory regions of a gene, which may be found at the 5' or 3' side of the coding region, or within the coding  
30 region, or within introns. Typically, a promoter is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The typical 5' promoter sequence is bounded at its 3' terminus by the  
35 transcription initiation site and extends upstream (5'

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direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence is a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

The term "nucleic acid construct" or "DNA construct" is sometimes used to refer to a coding sequence or sequences operably linked to appropriate regulatory sequences and inserted into a vector for transforming a cell. This term may be used interchangeably with the term "transforming DNA". Such a nucleic acid construct may contain a coding sequence for a gene product of interest, along with a selectable marker gene and/or a reporter gene.

The term "selectable marker gene" refers to a gene encoding a product that, when expressed, confers a selectable phenotype such as antibiotic resistance on a transformed cell.

The term "reporter gene" refers to a gene that encodes a product which is easily detectable by standard methods, either directly or indirectly.

A "heterologous" region of a nucleic acid construct is an identifiable segment (or segments) of the nucleic acid molecule within a larger molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, the heterologous DNA is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic

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sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

5           A "vector" is a replicon, such as plasmid, phage, cosmid, or virus to which another nucleic acid segment may be operably inserted so as to bring about the replication or expression of the segment. More specifically, the term "viral vector" refers to a virus  
10       that is able to transmit foreign or heterologous genetic information to a host. This foreign genetic information may be translated into a protein product, but this is not a necessary requirement for the foreign information.

          An "origin of replication" refers to those DNA  
15       sequences that participate in the in the initiation of DNA synthesis.

          A cell has been "transformed" or "transfected" by exogenous or heterologous DNA when such DNA has been  
20       introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to  
25       eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to  
30       establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth  
35       in vitro for many generations.

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The terms set forth below, relating to the biological molecules and methods of the present invention, are used throughout the specifications and claims.

5           The terms "immortalization" or "immortalized" refers to a cell, or a process for creating a cell, that will proliferate indefinitely in culture. In the present invention, immortalization refers to a process by which a primary cell culture is transformed in a way that causes  
10 the cells to behave in some respects like a tumor cell; specifically, in the proliferative characteristics of tumor cells.

          The terms "reverse-immortalization" refers to a process by which cells are immortalized by a means  
15 enabling them to be returned to their non-immortalized state at a later time.

          A "reversibly immortalized" cell is a cell that is presently in an immortalized state, but can be returned to a non-immortalized state at a later time,  
20 utilizing the reverse-immortalization process described herein.

          A "reverse-immortalized" cell is a cell that has been subjected to the entire process of reverse-immortalization, and now exists in a non-immortalized  
25 state.

          The term "suicide gene" refers to a gene that confers a lethality phenotype to cells which are reversibly immortalized. In more common terms, the "suicide gene" can be thought of as a negative selectable  
30 marker gene. Expression of its gene product enables the cell to be killed, i.e., by treatment of the cell with an exogenous agent such as an antibiotic or antiviral agent.

          The term "recombinase/recombinase target" refers to pairs of interacting molecules, one being a  
35 recombinase enzyme and the other being a DNA site

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specifically recognized and cleaved by that recombinase enzyme. The recombinase/recombinase target are paired by virtue of the specific interaction between the two, i.e., binding of the recombinase to its cognate DNA binding sequence, and cleavage of the DNA at that site.

## II. Description

In accordance with the present invention, a means is now available for minimizing or eliminating the risk of malignant transformation of transplanted hepatocytes which have been produced by immortalization of primary hepatocytes and expansion in cell culture. The inventors have reversibly immortalized hepatocytes using a recombinant retrovirus containing an oncogene capable of inducing tumorigenic growth, flanked by recombinase target sites. Excision of the oncogene from the immortalized cells is accomplished by site-specific recombination following introduction into the cells of a gene encoding the recombinase that specifically recognizes the recombinase target sites. After site-specific recombination and oncogene excision, cell proliferation stops and the cells develop the characteristics of differentiated hepatocytes. Moreover, the cells possess minimal oncogenic potential as determined by *in vitro* assays. These cells have been transplanted into hyperammonemia-induced encephalopathic rodents and were found capable of reversing the condition.

The reverse-immortalized hepatocytes described above are superior to those heretofore produced because the risk of their malignant transformation in transplant patients is greatly reduced. The safe use of these cells for hepatocyte transplantation has been even further augmented in accordance with the invention, by the addition of a "suicide" gene to the initial retroviral

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construct used to immortalize the cells. In an exemplary embodiment, the suicide gene is a herpes simplex virus thymidine kinase (HSV-tk) gene, incorporated into the retroviral vector between the two loxP sites. If the oncogene is successfully excised by the Cre recombinase via the mechanism described above, the HSV-tk gene also is excised. If it is not excised, the cell can be destroyed by treatment with gancyclovir, an antiviral agent that targets the HSV-tk gene product.

Thus, the reversibly immortalized hepatocytes of the invention are hepatocytes that comprise a heterologous DNA construct comprising a selectable marker gene and an oncogene that enables the cells to proliferate in culture, the selectable marker gene and the oncogene together being flanked by DNA binding sites for a recombinase. Optionally, the DNA segment further comprises a "suicide" gene, also disposed within the recombinase binding sites. The invention is practiced by transforming the primary hepatocytes with the DNA construct, culturing the transformed hepatocytes under standard conditions suitable to expand the population of transformed hepatocytes, then exposing the transformed hepatocytes to the recombinase that recognizes the binding sites on the DNA construct (e.g. by infecting the transformed hepatocytes with a viral vector containing a gene encoding the recombinase). If the DNA construct also contains a "suicide" gene, the hepatocytes are further subjected to the conditions that will kill any cells still containing the DNA construct, following treatment with the recombinase.

Primary hepatocytes may be obtained from any donor or source. Preferably, the donor or source is a mammal, such as a mouse, and most preferably, the donor or source is a human. Methods for obtaining and initiating cultures of primary hepatocytes are well known

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in the art.

Any oncogene may be used to reversibly immortalize primary hepatocytes, and many of these are known in the art. The SV40Tag gene (a known oncogene  
5 used to immortalize primary cells) is preferred for use, but others may be used. These include, but are not limited to, viral oncogenes as known in the art, and cellular oncogenes such as mutant p53 genes or c-met genes encoding hepatocyte growth factor receptor among  
10 others.

Similarly any selectable marker gene may be used in the DNA construct carrying the oncogene, and many of these are known in the art. For instance, several selectable marker systems are described in "Current  
15 Protocols in Molecular Biology", eds. Frederick M. Ausubel et al., John Wiley & Sons, 1999.

In addition many examples of suitable "suicide" genes are known in the art. The HSV-tk gene is preferred for use, but others may be used. Many examples are set  
20 forth by Ausubel et al., 1999, *supra*.

In addition to the immortalizing oncogene, selectable marker gene and the suicide gene, the DNA construct may comprise one or more desired genes, such as to promote growth or to provide a function reduced or  
25 missing from the donor's hepatocytes.

The aforementioned genes may be operably linked to one or more 5' and/or 3' expression-controlling regions, as is known in the art. With reference to promoters, constitutive or inducible promoters may be  
30 utilized, also as is known in the art.

DNA recombinase systems suitable for use in the invention are also known in the art. The cre/lox system (Cre recombinase, LoxP binding sites) is preferred for use, but other systems can also be used, including, but  
35 are not limited to the FLP/FRT system from *Saccharomyces*



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*cerevisiae*. It will be understood that if the DNA construct contains target binding sites for a particular recombinase, it is that recombinase that is to be used in reversing the immortalization of the hepatocytes.

5 United States Patent No. 5,629,159 to Anderson (incorporated by reference) describes a variety of DNA constructs exemplified for use in immortalization and dis-immortalization of pancreatic islet cells and neural cells. One or more of these variations may be adapted,  
10 in whole or in part, for reversible immortalization of hepatocytes in accordance with the present invention, using the methods described herein.

A preferred embodiment of the invention comprises (1) immortalizing primary hepatocytes with a  
15 retroviral vector containing the SV40Tag gene, the HSV-tk gene and a suitable selectable marker gene (e.g., *neo* or HSA, encoding the heat-stable antigen), flanked by loxP sites; (2) selecting transformants and growing them in culture; (3) reversing the immortalization by infecting  
20 the cells with an adenovirus vector carrying an expressible Cre recombinase gene to excise the oncogene; and, optionally, (4) destroying cells in which the oncogene was not successfully excised by treating the cells with gancyclovir. Vectors and systems of this type  
25 have been developed for reversible immortalization of various primary cells (Westerman & Leboulch, Proc. Natl. Acad. Sci. USA 93: 8971-8976, 1996), but have not been used for reversible immortalization of primary  
hepatocytes. More importantly, prior to the present  
30 invention, it was unknown whether such a system could be used to produce hepatocytes that would function *in vivo* following transplantation and that would be of sufficiently low oncogenic potential to be safe for such use.

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The preferred embodiment has been tested, as described in detail in the example. Briefly, hepatocytes were immortalized using a recombinant retrovirus containing the gene encoding SV40Tag flanked by loxP recombination target sites. Excision of SV40Tag from immortalized cells could then be accomplished by site-specific recombination with Cre-recombinase. Cells immortalized with this recombinant virus expressed SV40Tag and doubled in number every 48 hrs. After excision of the gene encoding SV40Tag with Cre-recombinase, cells stopped growing, DNA synthesis fell by 90%, and production of liver-specific mRNAs was either increased or became newly detectable. In addition, the morphology and epithelial cell polarity of the cells became more characteristic of differentiated hepatocytes. To determine their malignant potential, immortalized hepatocytes were transfected to express a second oncogene, activated H-ras. SV40Tag<sup>+</sup>/H-ras<sup>+</sup>-immortalized cells were capable of anchorage-independent growth and developed into tumors when injected in SCID mice. After SV40Tag excision using the Cre-recombinase, anchorage-independent growth stopped and tumor formation in SCID mice was abolished. Since immortalized hepatocytes also contained the gene encoding herpes simplex virus thymidine kinase, treatment with gancyclovir also produce complete regression of established tumors in mice.

Taken together, these results demonstrate that the reverse-immortalized hepatocytes produced by the method described above function *in vivo* as hepatocytes following transplantation. Moreover, the cells are of sufficiently low oncogenic potential to be safe for transplantation into patients in need of such treatment. Although similar reverse immortalization protocols have been employed for other cell types (e.g., pancreatic islet cells, muscle cells, neuronal cells), the

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successful reversal of immortalization to yield *in vivo* functional hepatocytes was an unexpected result. The aforementioned cell types were required to perform only one or two functions to be considered "functional"

5 following reverse immortalization. For instance, muscle cells provide structural support. The predominant function of pancreatic islet cells is to produce insulin. Neuronal cells are judged by their ability to provide a structural neural connection. In contrast, hepatocytes  
10 perform a plethora of functions, many or all of which must be present in order for the cell to function *in vivo*. Such functions include synthesis of key proteins and enzymes, regulation of carbohydrate and fat metabolism and detoxification of the blood, among others.

15 The inventors have provided evidence of differentiated hepatic function by the immortalized cells as evidenced by a variety of liver-specific functional proteins. The proteins represent a range of characteristics found only in differentiated hepatocytes.  
20 Albumin is uniquely produced by liver parenchymal cells and is a secreted protein. Androsterone-UGT is a microsomal protein that is expressed in differentiated hepatocytes only after birth and is an enzyme that degrades endogenous steroids. ASGPR, a plasma membrane  
25 protein, is lost in dividing cells and is the only liver specific protein whose expression is strongly controlled at the translational level.

Thus, the present invention demonstrates that, by transducing primary hepatocytes with a recombinant  
30 virus incorporating an oncogene, a suicide gene and a recombinase/recombinase target system, a well-differentiated, reversibly-immortalized non-tumorigenic hepatocyte cell line is generated. The successful generation of such a cell line would not have been  
35 predictable in advance of the results described in

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accordance with the present invention.

Hepatocyte transplantation holds great promise as an alternative to organ transplantation for patients with liver-based metabolic diseases and hepatic failure.

5 A significant limitation to the development of this therapy relates to the limited availability of hepatocytes for transplantation. Theoretically, factors that limit the availability of solid organs for transplantation should not affect the availability of

10 hepatocytes for this purpose. Primary hepatocytes have a tremendous capacity to proliferate *in vivo* and a small number of cells can be used to sequentially repopulate several generations of experimental animals whose liver cells are defective and can be replaced with unaffected

15 donor hepatocytes. In addition, isolated liver cells can be cryopreserved for use when needed. Unfortunately, techniques for expanding isolated human hepatocytes in tissue culture and for cryopreservation of human hepatocytes heretofore were not adequately developed to

20 be useful for human hepatocyte transplantation. Alternatives to the use of primary human hepatocytes for transplantation include the use of hepatocytes derived from other species and human hepatocytes conditionally immortalized for selective expansion in tissue culture.

25

The following example is provided to describe the invention in greater detail. It is intended to illustrate, not to limit, the invention.

30

**EXAMPLE 1**  
**Reversible Immortalization of Rat Primary**  
**Hepatocytes using the Cre/Lox System**

In the protocols described in this example, rat

35 primary hepatocytes were immortalized using a recombinant Moloney-based retrovirus containing the gene encoding

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SV40Tag flanked by loxP sites. Cells were characterized before and after treatment with a recombinant adenovirus capable of transferring the gene encoding the Cre-recombinase to determine whether this approach could produce an hepatocyte cell line that would be useful clinically for transplantation.

#### Materials and Methods:

**Animals.** Inbred male Lewis rats (150-250 g) were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and maintained in the Animal Resource Facility of the University of Nebraska College of Medicine (Omaha, NE). Animals were maintained on standard laboratory chow on a 12-hour light/dark cycle. Severe combined immunodeficiency (SCID) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in a barrier facility at the University of Nebraska College of Pharmacy. All procedures performed were approved by the University of Nebraska Institutional Animal Care and Use Committee and thus within the guidelines for human care of laboratory animals.

**Recombinant Virus and Producer Cell Lines.** The recombinant Moloney-based retrovirus SSR69 (Fig. 1), which contains the SV40Tag, herpes simplex virus thymidine kinase, and hygromycin resistance genes flanked by loxP sites has been described previously (Westerman KA, Leboulch P, Proc Natl Acad Sci U S A 1996; 93:8971-6). The SSR69 retrovirus producer cell line was maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco/BRL, Gaithersburg, MD) containing 10% newborn calf serum, 320 µg/ml Hygromycin B, and 1% penicillin/streptomycin (Gibco/BRL, Gaithersburg, MD) and produces a virus titer of  $1 \times 10^4$  hygromycin-resistant cfu/ml when assayed on NIH 3T3 cells.

A recombinant adenovirus containing the gene

- 20 -

encoding Cre-recombinase (Ad-Cre) was obtained. The transduction activity and titer of purified Ad-Cre virus stock was determined by counting G418-resistant cell colonies after infection of SSR69 producer cells with serial dilutions of virus stock.

**Measurement of albumin production.** To determine albumin production by immortalized hepatocytes, cells were plated into 12-well plates and incubated at 37°C and 5% CO<sub>2</sub> in 0.3 ml/well of chemically-defined HGM media (Block GD, Locker J, Bowen WC, et al., J Cell Biol 1996; 132:1133-49). After 48 hours, the media was collected and assessed for albumin production by enzyme-linked immunosorbent assay (ELISA). ELISA was performed using a rabbit polyclonal anti-rat albumin capturing antibody (Cappel, Durham, NC) and a rabbit peroxidase-conjugated anti-rat albumin (Cappel, Durham, NC) secondary antibody. The standard curve was constructed using purified rat albumin (fraction V) purchased from Sigma Chemical Co. (St. Louis, MO).

**Isolation and Immortalization of Lewis Rat Hepatocytes.** Hepatocytes from Lewis rats were isolated by in situ collagenase (type I, Worthington Biochemical Corporation, freehold, NJ) perfusion and plated on tissue culture flasks in Immortalization Medium [IM; DMEM containing 4% fetal calf serum, 0.2 µM Dexamethasone (Sigma Chemical Co., St. Louis, MO), and 1% penicillin/streptomycin supplemented with 10 µg/ml epidermal growth factor (EGF). After incubation for 24 hrs at 37°C and 5% CO<sub>2</sub>, hepatocytes were transduced with the SSR69 retrovirus. Two days after infection, the EGF was removed and hygromycin was added to the culture media at 200 µg/ml. Hygromycin-resistant colonies emerged in 3 weeks. Individual colonies were isolated using cloning rings and expanded in culture at 37°C and 5% CO<sub>2</sub>.

**<sup>3</sup>H-Thymidine uptake.** Primary hepatocytes and immortalized hepatocyte clones were cultured on Primaria

(Becton Dickinson Labware, Lincoln Park, New Jersey) plastic tissue culture plates at 37°C and 5% CO<sub>2</sub> at a density of 3 x 10<sup>4</sup> cells per well in 6-well plates. <sup>3</sup>H-Thymidine incorporation was measured as previously described (Fox IJ, Chowdhury NR, Gupta S, et al. Hepatology 1995; 21:837-46.). Briefly, cells were incubated with <sup>3</sup>H-thymidine (5 µCi/ml) at 37°C for 2 hrs. <sup>3</sup>H-thymidine incorporation by primary hepatocytes was studied under the same conditions for comparison. The labeling medium was removed, the cells were washed with PBS and released with trypsin. Cells were then counted and trichloroacetic acid precipitable radioactivity was determined by scintillation counting using Hydrofluor (National Diagnostics, Manville, N.J.).

**Determination of cell number:** Primary hepatocytes and immortalized hepatocyte clones were plated at a density of 3 x 10<sup>4</sup> cells per well in 6-well plates and cultured at 37°C. At 24 hr intervals, cells were released with trypsin and stained with trypan blue. Cell counts were determined using a hemocytometer

**RNA isolation and semi-quantitative reverse transcription-polymerase chain reaction (SQ-RT-PCR).**

Total RNA was isolated by using Trizol reagent (Gibco/BRL, Gaithersburg, MD) according to the manufacturer's recommendations. Expression of mRNA was analyzed by SQ-RT-PCR as described previously (Cai J, Phelan SA, Hill AL, Loeken MR. Diabetes 1998; 47:1803-5). Briefly, 200 ng of total RNA was reverse transcribed with random hexamer primers and serial dilutions of the resulting cDNA were amplified by PCR using sequence-specific primers.

**SCID mouse studies.** The tumorigenicity of cell lines was assayed by subcutaneous injection of cells into the flanks of immunodeficient (SCID) mice. Cells were dislodged from monolayer culture with trypsin, washed,

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and suspended in PBS. SCID mice were inoculated subcutaneously with  $1 \times 10^6$  cells per site. Animals were monitored every third day for the development of growth at the sites of injection. Animals were sacrificed at 24 weeks or when tumors reached 1 to 2 cm in diameter.

Tumors were processed for routine histology and immunohistochemistry. To determine tumor sensitivity to treatment with gancyclovir, some animals were given a 0.2 ml intraperitoneal injection of gancyclovir (50 mg/kg) daily for 14 days when tumors reached 1-1.2 cm in size.

Immunohistochemistry was employed to determine SV40Tag expression in tumors. Unfixed frozen tumor sections were fixed in 1:1 acetone:methanol at  $-20^\circ\text{C}$  for 20 min. After incubation in blocking buffer (PBS + 1% BSA), sections were incubated with mouse anti-SV40Tag mAb (CalBiochem, La Jolla, CA) followed by goat DTAF-conjugated anti-mouse IgG (Accurate Chemical and Scientific Co, Westbury, NY).

**Soft agar assay.** To assay the capacity for anchorage independent growth,  $5 \times 10^4$  cells were suspended in 2 ml of 0.3% Difco agar in IM and gently overlaid onto 60 mm dishes containing a lower layer of 0.5 % agar. Cultures were fed every 5 to 7 days with a small amount of media. After 20 days of growth at  $37^\circ\text{C}$ , the dishes were stained with 0.5 mg/ml p-iodonitrotetrazolium violet (Sigma Chemical Co., St. Louis, Mo), and macroscopically visible colonies ( $>100 \mu\text{m}$  in diameter) were scored. HepG2 cells were used as a positive control.

**Production of activated H-ras immortalized hepatocytes.** To establish an SV40Tag<sup>+</sup>/H-ras<sup>+</sup>-expressing immortalized hepatocyte cell line, a plasmid encoding activated H-ras and neo resistance, pSV2-Neo-EJ, was transfected into the SV40Tag immortalized hepatocytes by  $\text{CaPO}_4$  precipitation. Two days later, transduced cells were selected in G418 (800  $\mu\text{g/ml}$ ). The resultant G418-resistant cells were then subcloned using cloning rings.



**Results:****Establishment of immortalized cell lines.**

Following SSR69 retrovirus infection, transduced hepatocytes expressed SV40Tag, hygromycin resistance, and contained the gene encoding Herpes Simplex Virus thymidine kinase (HSV-tk). Hygromycin-resistant colonies emerged in 3 weeks and individual colonies were isolated using cloning rings. Fifty cell clones were obtained and at least partially characterized. To determine which immortalized cell clones retained characteristics of differentiated hepatocytes, individual cell lines were screened by ELISA for secretion of albumin in the culture media. Twenty-four of the original 50 cell lines produced albumin when initially isolated.

The albumin-producing cell lines were then further characterized for additional evidence of hepatocyte-specific gene expression by RT-PCR using primers for the asialoglycoprotein receptor (ASGR), uridine diphosphate-glucuronosyltransferase-2 (UGT2), and hepatocyte nuclear factor 4 (HNF4). Eleven clones expressed only albumin mRNA, seven expressed ALB and one other liver-specific mRNA, three expressed 3 liver-specific mRNAs and three clones expressed all 4 mRNAs. Based on high-level albumin production and expression of all liver-specific mRNAs, one cell line (C8-B) was subcloned and used for further investigation.

**Excision of SV40Tag by site-specific deletion.**

To determine whether the gene encoding the SV40Tag could be efficiently deleted from immortalized C8-B cells, the cells were infected with Ad-Cre at an MOI of 1. Genomic DNA and mRNA were isolated at various time points after infection and analyzed by PCR and RT-PCR using SV40Tag/SSR69 specific primers (Fig. 1). As shown in Fig. 2, the DNA fragment encoding the SV40Tag was completely excised by the Cre recombinase within 2 days

- 24 -

of Ad-Cre infection. SV40Tag mRNA expression was ultimately eliminated; however, low level gene expression could still be detected up to 5 days after Ad-Cre infection. These results indicate that the gene encoding SV40Tag in SSR59-immortalized hepatocytes can be efficiently excised immediately after transduction with the Cre recombinase and that gene expression can be completely shut down using Cre/loxP-mediated site-specific recombination.

10           **Cell Morphology.** Light microscopy of the cultured immortalized cells was performed using phase contrast. Treatment with Cre recombinase changed the morphology of the C8-B cells. Six days after Ad-Cre infection, C8-B cells enlarged and increased their cytoplasm to nucleus ratio. Transmission electron  
15           microscopy of Ad-Cre treated and untreated cells revealed abundant mitochondria, lysosomes, rough endoplasmic reticulum and smooth endoplasmic reticulum. However, intercellular junctions with microvilli characteristic of  
20           bile canaliculi were seen in Ad-Cre treated cells only.

**Cell proliferation and DNA synthesis.** Before Ad-Cre treatment, immortalized cells doubled in number in approximately 48 hrs. After Ad-Cre infection, neither C8-B nor cultured primary hepatocyte cell numbers  
25           significantly increased (Fig. 3A).

              Fig. 3B shows <sup>3</sup>H-thymidine incorporation by cultured immortalized hepatocytes before and after Ad-Cre infection. The incorporation of <sup>3</sup>H-thymidine by cells before Ad-Cre infection was 15-fold greater than that by  
30           freshly cultured primary hepatocytes (p< 0.01 by Student's t-test). Three days after Ad-Cre infection, DNA <sup>3</sup>H-thymidine incorporation was reduced to 1.5 fold that of primary hepatocytes which had been cultured for  
35           24 hours.

- 25 -

**Liver-specific mRNA expression.** To determine whether loss of the SV40Tag by site-specific recombination would produce a more differentiated phenotype, C8-B cells were assayed by SQ-RT-PCR before and after Ad-Cre infection for expression of liver specific mRNAs. Total cellular RNA was isolated before and 2, 5, and 7 days after Ad-Cre infection and reverse transcribed using random hexamer primers. The resulting cDNAs were then serially diluted and PCR amplified using sequence-specific primers for ALB (a secretory protein), ASGR (a cell surface protein), UGT1 (a microsomal protein responsible for glucuronidation of bilirubin), UGT2 (a microsomal protein responsible for glucuronidation of steroids), and HNF4 (a transcription factor). RNA extracted from isolated primary rat hepatocytes was used as a positive control, and actin mRNA was used as an RNA loading control. As shown in Fig. 4, the mRNA level of all liver-specific proteins was increased. By day 5 to 7 after Ad-Cre infection, ALB, HNF4 and UGT1 gene expression was detectable at a higher level than before Cre recombinase treatment, and UGT2 and ASGR mRNAs became newly detectable.

**Malignant potential of SSR69 immortalized hepatocytes.** The malignant potential of C8-B cells was first determined by using a soft agar assay. The result showed that C8-B cells produced no anchorage independent colonies, but grew as small clusters that expanded in only two dimensions. This contrasts markedly with the growth pattern of HepG2 cells that grew into large 3-dimensional colonies and continued to enlarge exponentially with time. The malignant potential of C8-B cells, which still produce SV40Tag, was further analyzed by transplanting them into the flanks of SCID mice. Subcutaneous implantation of  $1 \times 10^6$  cells produced no tumors after 24 weeks of implantation, whereas injection

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of the same number of HepG2 cells produced large tumors within 4 weeks of implantation (data not shown).

Since transfection of SV40 immortalized hepatocytes with activated c-Ha-ras has been shown to  
5 transform them into strongly tumorigenic cells which are histologically poorly differentiated, C8-B cells were transduced to express the activated ras gene in order to assess whether mutational activation by a second  
10 transforming gene could produce malignant transformation of SSR69-immortalized cells. Cells were transfected with plasmid pSV2-Neo-EJ and individual G418-resistant cell colonies were cloned and analyzed for expression of H-ras and SV40Tag genes by RT-PCR. One cell line, which  
15 expressed both H-ras and SV40Tag (C8-B/Ras), was used for further study.

As shown in Fig. 5, C8-B/Ras cells developed anchorage-independent large colonies in soft agar. Since these cells contain the gene encoding the herpes simplex virus thymidine kinase, colonies were assessed for  
20 sensitivity to treatment with gancyclovir and could be eliminated in media containing 5  $\mu$ M gancyclovir. Following infection with Ad-Cre, soft agar culture produced only rare three-dimensional colonies. These colonies resulted from failure to undergo site-specific  
25 recombination since all were sensitive to gancyclovir and, as determined by PCR, still contained the genes encoding the SV40Tag and H-ras (data not shown).

Tumor potential was further assessed by inoculating C8-B/Ras cells into SCID mice. C8-B/Ras  
30 cells induced tumors at all inoculated sites, and grew to more than 1 cm within 3 weeks. Following SV40Tag excision with Ad-Cre, tumors developed in only one of four inoculation sites and reached 1.5 cm in size only after 8 weeks. Immunohistochemistry showed that this  
35 tumor grew from cells which had not undergone

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recombination since all tumor cells stained positively for SV40Tag (data not shown).

When tumor-bearing animals were given a 14-day course of gancyclovir (50mg/kg), tumors (> 1 cm in size) stopped growing within 5 days of beginning therapy, and could no longer be identified clinically or histologically by the end of treatment. In addition, no tumor recurrence occurred during a 4-week post-gancyclovir observation period.

#### Discussion:

We have previously demonstrated that primary rat hepatocytes, immortalized with a thermolabile mutant SV40 large T antigen can maintain a significantly differentiated hepatic phenotype in culture and can function as well as primary hepatocytes following transplantation in animal models of liver-based metabolic disease and liver failure. Those cells did not demonstrate anchorage independent growth in tissue culture and, when transplanted into syngeneic rats or immunodeficient mice, did not form tumors. Down-regulation of the mutant thermolabile large T antigen at physiologic temperature provided a minimal degree of protection from the development of tumors in animals receiving the transplants. However, a variety of factors could potentially affect the function and growth of such cells.

To provide more stringent control over expression of the transforming gene, the loxP/Cre system was employed. In this study, the gene encoding the SV40Tag was completely eliminated from C8-B cells within two days of Ad-Cre infection. After the gene was deleted, the cells stopped growing, their DNA synthesis fell, their expression of liver-specific mRNAs increased, and they regained the morphological appearance of

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differentiated hepatocytes. Thus, reversal of immortalization was accomplished with complete removal of the offending transforming gene.

While C8-B cells were not tumorigenic by *in vitro* assay or following transplantation, when transduced to express a second transforming gene, SV40Tag<sup>+</sup>/H-ras<sup>+</sup> cells formed large colonies in soft agar and developed into tumors in SCID mice. After Ad-Cre infection, only occasional SV40Tag<sup>+</sup>/H-ras<sup>+</sup> C8-B cells formed anchorage-independent cell colonies in soft agar and produced slow growing tumors in SCID mice. These colonies and tumors, however, were sensitive to treatment with the antiviral agent gancyclovir. Since the gene encoding HSV-tk in the immortalized cells is flanked by loxP sites, SV40 Tag-deleted cells are not sensitive to gancyclovir. Therefore, gancyclovir could be administered to transplant recipients to eliminate engrafted SV40 Tag-expressing immortalized hepatocytes but would not eliminate engrafted SV40 Tag-deleted cells in recipients treated with gancyclovir. The recombinant vector used to make the immortalized hepatocytes in these experiments produced cells which express the neomycin-resistance gene following recombination. This vector could easily be redesigned so that transduced cells express green fluorescent protein upon recombination. Thus, it would be possible to select cells for transplantation which have undergone recombination based on their fluorescence characteristics.

The present invention is not limited to the embodiments described above, but is capable of variation and modification without departure from the scope of the appended claims.

What is claimed:

1. A method of making a population of functional hepatocytes for transplantation into a patient, which comprises:

- 5                   a) providing a sample of primary hepatocytes;
- b) immortalizing the hepatocytes by transforming the hepatocytes with a vector comprising a removable DNA segment containing an oncogene, thereby
- 10                  producing immortalized hepatocytes;
- c) growing the immortalized hepatocytes; and
- d) removing the oncogene from the immortalized hepatocytes, the removal resulting in the production of the population of functional hepatocytes
- 15                  for transplantation into the patient.

2. The method of claim 1, wherein the hepatocytes are obtained from a human donor.

- 20                  3. The method of claim 1, wherein the oncogene is made removable by flanking it with recombinase target sites, and the removing is accomplished by introducing into the immortalized cells a gene that is expressed to produce a recombinase that specifically recognizes the
- 25                  recombinase target sites.

4. The method of claim 3, wherein the recombinase is Cre recombinase and the recombinase target sites are loxP sites.

- 30                  5. The method of claim 1, wherein the oncogene is a gene encoding SV40 large T antigen.

6. The method of claim 1, wherein the removable DNA segment further contains a suicide gene, which encodes a gene product that enables destruction of
- 35                  removable DNA segment further contains a suicide gene, which encodes a gene product that enables destruction of

the immortalized cells by an exogenous agent if the removable DNA segment is not removed from the cells.

5           7. The method of claim 6, wherein the suicide gene is a gene encoding herpes simplex virus thymidine kinase, and the cells are destroyed by exposure to gancyclovir if the removable DNA segment is not removed from the cells.

10           8. A population of functional hepatocytes produced by the method of claim 1.

15           9. A method of treating a patient for hepatic failure, comprising transplanting into the patient a sufficient quantity of the hepatocytes of claim 8 to provide hepatic function to the patient.

20           10. A method of making a population of functional hepatocytes for transplantation into a patient, which comprises:

          a) providing a sample of primary hepatocytes;

25           b) immortalizing the hepatocytes by transforming the hepatocytes with a vector comprising a removable DNA construct containing an oncogene, a selectable marker gene, and a gene encoding herpes simplex virus thymidine kinase, the genes together being flanked on either side by loxP sites;

30           c) growing the immortalized hepatocytes; and  
          d) reversing the immortalization of the hepatocytes by removing the DNA construct from the immortalized hepatocytes, the removing being accomplished by introducing into the immortalized hepatocytes a gene encoding Cre recombinase to effect excision of the DNA  
35           construct at the loxP sites, the excision resulting in the production of the population of functional hepatocytes for transplantation into the patient.



11. The method of claim 10, wherein the hepatocytes are obtained from a human donor.

12. The method of claim 10, wherein the  
5 oncogene is a gene encoding SV40 large T antigen.

13. The method of claim 10, wherein the selectable marker gene confers hygromycin resistance to cells expressing the gene.

10

14. A population of functional hepatocytes produced by the method of claim 10.

15. A method of treating a patient for hepatic  
15 failure, comprising transplanting into the patient a sufficient quantity of the hepatocytes of claim 14 to provide hepatic function to the patient.

16. An immortalized hepatocyte comprising a  
20 primary hepatocyte transformed with a DNA construct comprising two recombinase target sites that flank an oncogene which confers immortalization to the hepatocyte, wherein the immortalization is reversible by excision of the DNA construct by cleavage at the recombinase target  
25 sites when the target sites are exposed to a recombinase that specifically recognizes the target sites.

17. The immortalized hepatocyte of claim 16,  
wherein the recombinase target sites are loxP sites and  
30 the immortalization is reversible by Cre recombinase cleavage at the loxP sites.

18. The immortalized hepatocyte of claim 16,  
wherein the DNA construct further comprises a selectable  
35 marker gene.

19. The immortalized hepatocyte of claim 16,  
wherein the DNA construct further comprises a suicide  
gene, which encodes a gene product that enables  
destruction of the immortalized hepatocyte by an  
5 exogenous agent if the DNA construct is not removed from  
the cells.

20. The immortalized hepatocyte of claim 19,  
wherein the suicide gene is a gene encoding herpes  
10 simplex virus thymidine kinase, and the exogenous agent  
is gancyclovir.

21. The immortalized hepatocyte of claim 16,  
wherein the hepatocyte is obtained from a human donor.  
15

22. The immortalized hepatocyte of claim 20,  
wherein the hepatocyte is obtained from a rat donor.

23. A cell line comprising a population of the  
20 immortalized hepatocyte of claim 22, which is cell line  
C8-B.

24. A reverse-immortalized hepatocyte that is  
functional upon transplantation into a patient, produced  
25 by exposing the DNA construct within the immortalized  
hepatocyte of claim 16 to a recombinase that excises the  
DNA construct by cleavage at the recombinase target  
sites.

30 25. A method of treating a patient for hepatic  
failure, comprising transplanting into the patient a  
sufficient quantity of the reverse-immortalized  
hepatocytes of claim 24 to provide hepatic function to  
the patient.

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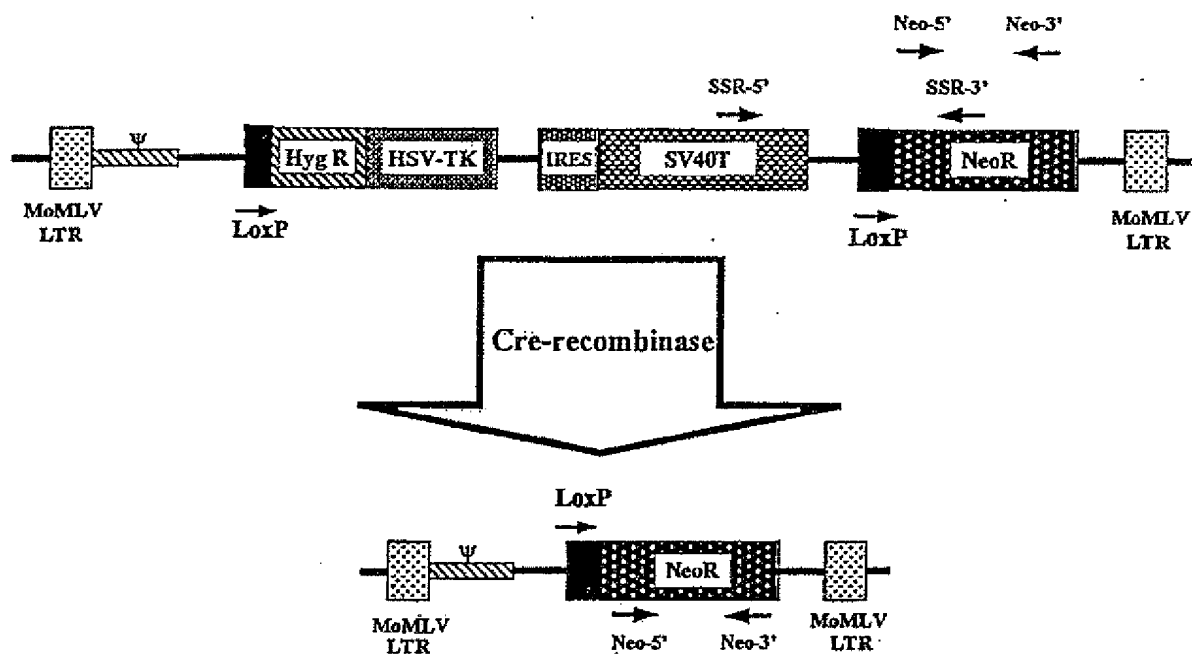
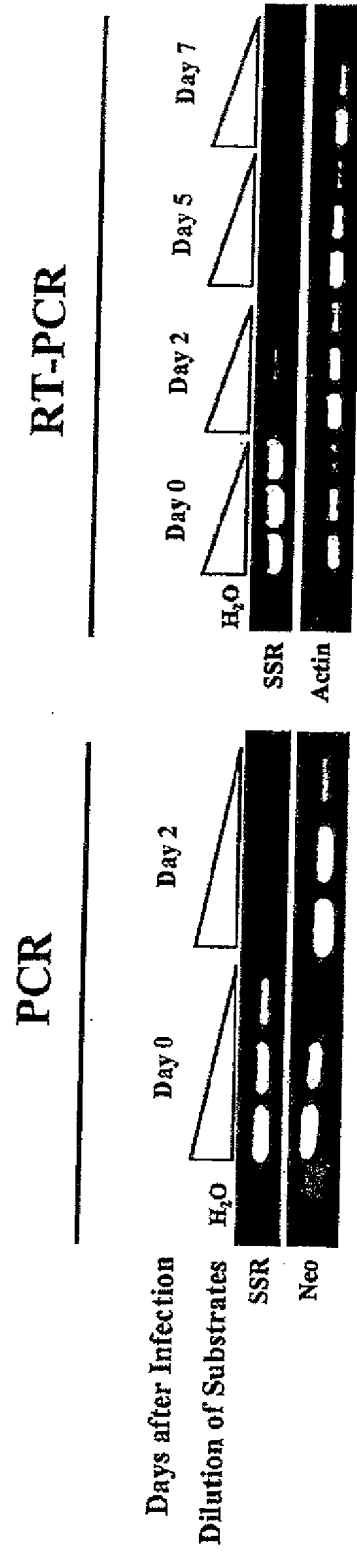


Fig. 1

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**Fig. 2**

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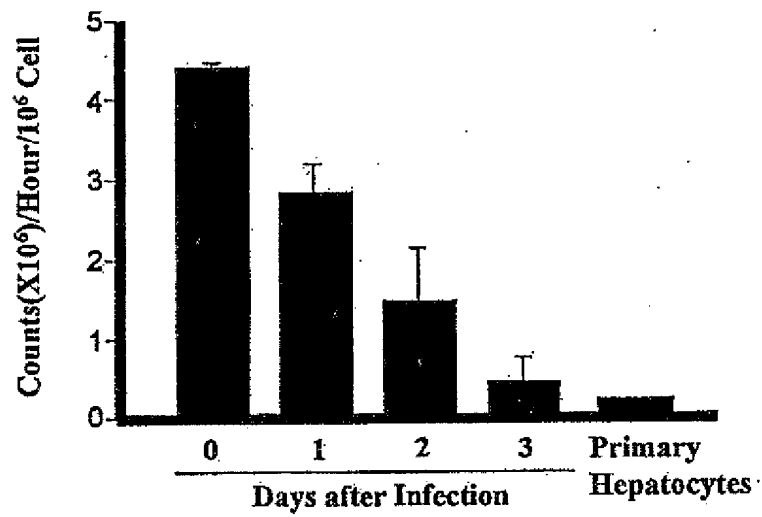
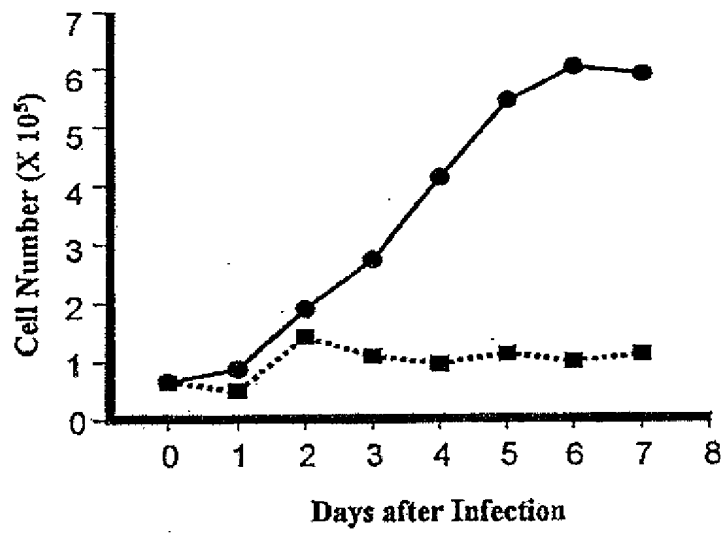
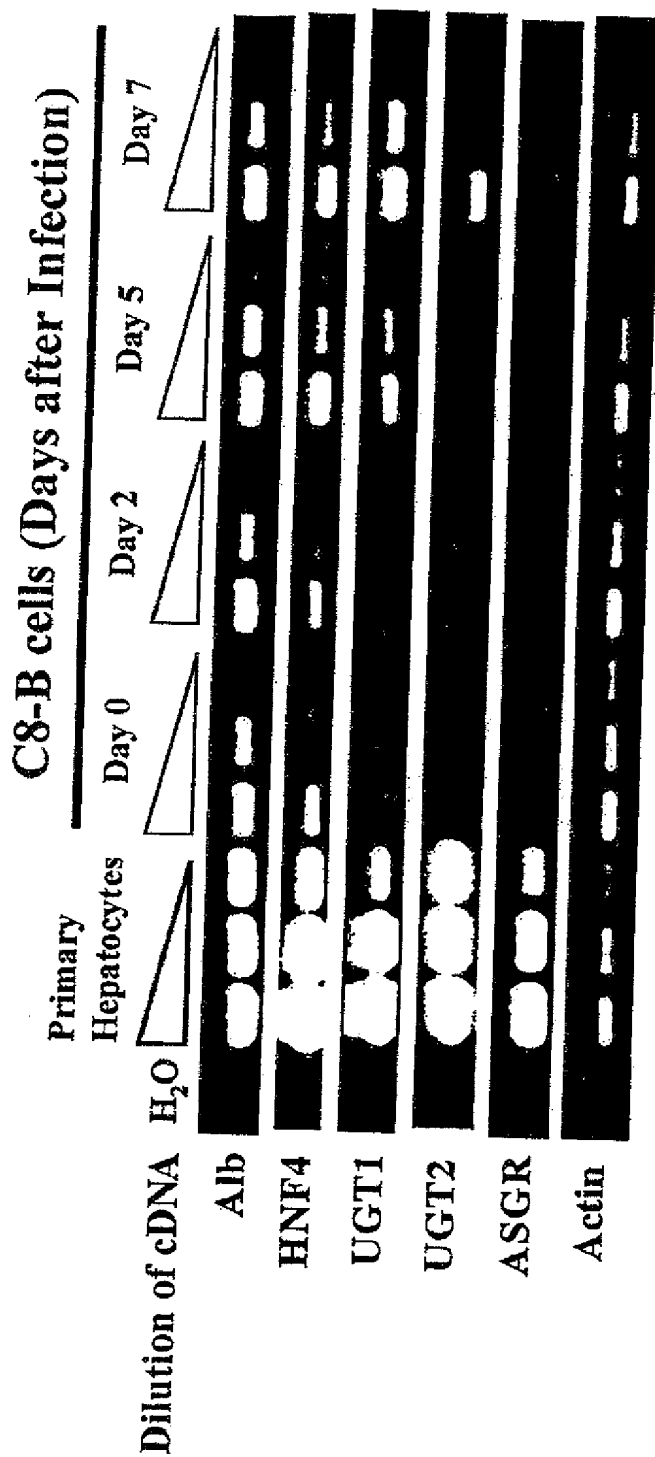


Fig. 3

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**Fig. 4**

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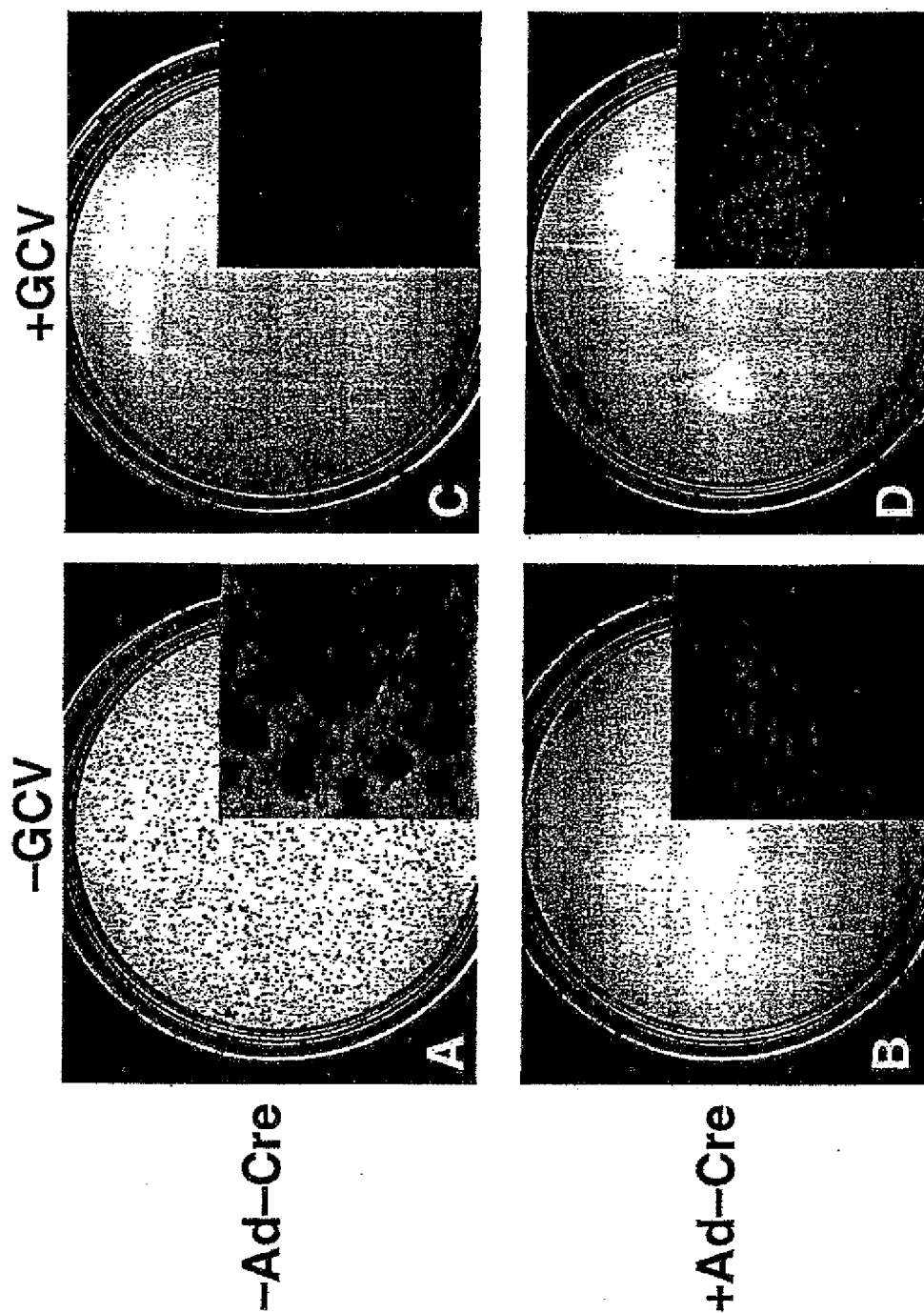


Fig. 5

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/22207

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A01N 63/00; C12N 5/10, 15/09

US CL : 424/93.1, 93.2; 435/320.1, 325

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.1, 93.2; 435/320.1, 325

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WESTL.2, CAPLUS, MEDLINE, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97/32972 A1 (RHODE ISLAND HOSPITAL) 12 September 1997, claims 1, 4, and 5.	1, 2, 5, 8, 9
Y		10-15
Y	WESTERMAN et al. Reversible immortalization of mammalian cells mediated by retroviral transfer and site-specific recombination. Proceedings of the National Academy of Sciences of the United States of America. 20 August 1996, Vol. 93, No. 17, pages 8971-8976, especially abstract.	3, 4, 17

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

"A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"Z"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

05 DECEMBER 1999

Date of mailing of the international search report

03 FEB 2000

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/22207

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	Database MEDLINE, Accession Number 1999321299, KAWASHITA et al. 'Regression of hepatocellular carcinoma in vitro and in vivo by radiosensitizing suicide gene therapy under the inducible and spatial control of radiation', abstract, Human Gene Therapy. 10 June 1999, Vol. 10, No. 9, pages 1509-1519, especially abstract.	1, 2, 6, 7, 16, 19, 20
X	NAKAMURA et al. Treatment of surgically induced acute liver failure by transplantation of conditionally immortalized hepatocytes. Transplantation. 15 June 1997, Vol. 63, No. 11, pages 1541-1547, especially abstract.	1, 2, 5, 8, 9
Y		10-16, 21-5

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